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THE BACTERICIDAL ACTIVITY OF THE SERUM OF HEALTHY BEAGLES

by

G. H. MEADE
AND W. E. CLAPPER

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ABSTRACT

A relatively simple method for testing the bactericidal activity of serum from beagles which gave reproducible results was found. Activity was measurable for a strain of Escherichia coli, but not for Pseudomonas aeruginosa, Proteus mirabilis, or Bacillus subtilis. The E. coli bactericidin was inactivated by heat, was not restored by the addition of guinea pig complement, nor augmented by adding complement to the unheated serum. Such serum activity is apparently part of the immune mechanism of beagles and may be important in relation to their ability to resist invasion of the blood stream by intestinal bacteria.

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TABLE OF CONTENTS

LIST OF TABLES

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INTRODUCTION

The normal bactericidal activity of rabbit serum against Bacillus subtilis was reported by Marcus and Donaldson[1] to be decreased following irradiation. The decrease was greatest during the period when the animals were more susceptible to infection. Irradiation has also been shown to decrease the serum bactericidins in mice, rabbits, and guinea pigs to a strain of Escherichia coli[2]. These investigators did not feel that the decrease was related to the post-irradiation bacteremia in mice. Animal species differ in the activity of their serum to different bacteria. Tilden[3] found that dog serum was bactericidal only to Proteus morganii, Proteus X19, and Sarcina lutea when a large variety of bacteria, including E. coli, were tested. Mackie and Finkelstein[4] reported on the serum bactericidins against Gram positive and Gram negative bacteria in various animals. The literature on this subject has been reviewed by Skarnes and Watson[5].

As a part of a program to study the biological effects in beagles of inhalation of fission products, observation of any changes in their immune mechanisms seemed necessary. The bactericidal activity of the serum is one of these. This investigation was, therefore, undertaken to determine what bacterium could be used as a test organism for the factor in the beagle and what method would be most useful for processing a relatively large number of specimens quickly. The methods used to measure the serum activity, the bacteria tested, and some of the properties of the bactericidin are described.

METHODS

1. Organisms

A strain each of Pseudomonas aeruginosa, Proteus mirabilis, Bacillus subtilis, and Escherichia coli (ATCC 4157) were used as the test organisms. After growth in 10 ml of dextrose broth (Difco) for 24 hours at 37°C, the cultures were centrifuged, the broth poured off, and the bacteria suspended in distilled water to a density which gave a reading of 150 on the Klett colorimeter using a blue filter. Further dilutions were made, as found by previous trials with each bacterium, to make an easily determined colony count by the method described below.

2. Serum

Blood from healthy beagles was obtained from the Project colony. The serum, separated after the blood had clotted, was either used immediately or stored at 4°C for no longer than 24 hours.

3. Test

The procedure was essentially that described by Pindak[6] with minor modifications. Five-tenths ml of serum, 0.16 ml of bacterial suspension, and 0.32 ml of Kolmer's saline were mixed in a small screw-capped tube. From this tube duplicate 0.1-ml samples were immediately removed and spread evenly with a bent glass rod over the surface of desoxycholate or blood agar plates, depending upon the type of bacteria used. Further dilutions of 1-10 and 1-100 were made in saline and plated in a similar manner. The original undiluted bacterial suspension with serum was then incubated in a 37°C water bath for six hours and 0.1 ml of undiluted and 1-10 and 1-100 dilutions were again plated. The 6-hour incubation period had been determined by preliminary tests to be the most satisfactory. All plates were incubated overnight at 37°C, after which colonies were counted.

The activity after heating the serum to 56°C for 30 minutes and after the addition of guinea pig complement was determined by the method described above, except that the amount of serum was reduced to 0.25 ml,

the 10^{-3} bacterial suspension to 0.08 ml, and 0.16 ml of commercial guinea pig complement replaced the saline used in other experiments. Both heated and unheated complement were added.

4. Other Methods

(1) The turbidity of a broth suspension of E. coli and a measured amount of undiluted serum was determined at intervals with a Klett colorimeter.

(2) Filter paper disks were saturated with serum and placed on agar plates which had been inoculated with E. coli.

RESULTS

1. Ps. aeruginosa, Pr. mirabilis, B. subtilis

Sera from six dogs were incubated with the three test organisms. Plate counts were made in duplicate. The total number of bacteria in 0.1 ml of the suspensions before incubation may be seen in Table 1 in the first three columns under Pseudomonas aeruginosa. With no dilution, the colonies were too numerous to count. With a 1-10 dilution, approximately 400 colonies were found on every plate. The 1-100 dilution could be accurately counted. The absence of bactericidal activity may be seen by comparing the number of bacteria (as shown by the colonies on the plates) after incubation with the sera for six hours. The values are given in the three columns under the six-hour heading. The number of colonies shown on the plates inoculated with the 1-10 and 1-100 dilutions of the bacterial suspension indicates that there was an increase in growth. Similar results may be seen for the activity of the sera against Proteus mirabilis and Bacillus subtilis. There was no bactericidal activity for any of the three organisms. Myrvik and Weiser[7] have reported dog sera to have low activity against B. subtilis.

TABLE 1

ABSENCE OF BACTERICIDAL ACTIVITY OF SERUM FROM BEAGLES FOR *PSEUDOMONAS*, *PROTEUS*, AND *BACILLUS* SPECIES

	Number of colonies per plate*																	
	Pseudomonas aeruginosa						*Proteus mirabilis*						*Bacillus subtilis*					
	Before incub.			After 6-hrs.			Before incub.			After 6-hrs.			Before incub.			After 6-hrs.		
Dog number	0	1-10	1-100	0	1-10	1-100	0	1-10	1-100	0	1-10	1-100	0	1-10	1-100	0	1-10	1-100
B9	inn[a]	400+[b]	49	inn	inn	250	inn	300+	62	inn	inn	400+	inn	186	19	inn	400+	210
	inn	400+	58	inn	inn	224	inn	300+	58	inn	inn	400+	inn	201	24	inn	400+	200
B12	inn	400+	67	inn	inn	400+	inn	150	19	inn	inn	400+	inn	196	16	inn	inn	inn
	inn	400+	68	inn	inn	400+	inn	160	14	inn	inn	400+	inn	215	18	inn	inn	inn
B13	inn	400+	87	inn	inn	260	inn	140	15	inn	inn	300+	inn	175	15	inn	400+	180
	inn	400+	68	inn	inn	280	inn	160	18	inn	inn	300+	inn	188	16	inn	400+	196
B14	inn	400+	86	inn	inn	300+	inn	150	10	inn	inn	300+	inn	150	13	inn	inn	400+
	inn	400+	89	inn	inn	300+	inn	155	12	inn	inn	300+	inn	156	16	inn	inn	400+
B15	inn	400+	88	inn	inn	400+	inn	140	13	inn	inn	300+	inn	180	16	inn	400+	150
	inn	400+	74	inn	inn	400+	inn	136	18	inn	inn	300+	inn	184	18	inn	400+	139
B18	inn	400+	74	inn	inn	300+	inn	128	9	inn	inn	300+	inn	166	14	inn	inn	400+
	inn	400+	70	inn	inn	300+	inn	136	6	inn	inn	300+	inn	178	15	inn	inn	400+

Experimental conditions: 0.5 ml serum, 0.16 ml bacterial suspension diluted 10^{-3}, 0.32 ml Kolmer's saline.

*Inoculated with 0.1 ml. [a]inn = innumerable. [b]400+ and 300+ = approximate values.

2. E. coli

In preliminary testing, 36 specimens taken from 12 dogs at different times were found to be bactericidal to E. coli in most cases. The 1-100 dilution added nothing to the data that was not better demonstrated by the undiluted and 1-10 dilution, so this dilution was not included in the final studies. Plate counts showing the antibacterial activity of the sera from 12 dogs are listed in the four columns under "unheated serum" in Table 2. All sera appreciably reduced the number of bacteria after incubation for six hours.

Table 2 also shows that the bactericidal activity was destroyed by heating the sera to 56°C for 30 minutes. This is similar to the findings of Waisbren and Brown[8] that heating human sera to this temperature markedly reduced its bactericidal activity for E. coli. They also reported that, although the addition of guinea pig serum did not restore bactericidal activity to heated sera, it did enhance the activity in seven of nine of the unheated sera tested. Rowley[9] reported that mouse serum requires about 50% added guinea pig serum to show antibacterial activity against E. coli because mouse serum not only lacks complement, but is also anticomplementary.

These results prompted us to determine the effect of adding guinea pig serum to several of the dog sera. Both heated and unheated commercial guinea pig serum (complement) were added. The results in Table 3 indicate that guinea pig complement does not potentiate the bactericidin for E. coli in dog serum. Each of the sera or pools of sera were tested on different days which accounts for the variability of the initial colony counts.

It is well known that the bactericidal activity of many animal sera against Gram negative organisms is dependent upon a heat labile substance and that the activity of the heated serum may often be restored by the addition of the heat labile protein complex in serum called complement. In order to determine whether the bactericidin with which we were dealing was one of this type, three active dog

TABLE 2

BACTERICIDAL ACTIVITY OF SERUM FROM BEAGLES FOR E. COLI AND THE EFFECT OF HEAT

		Number of colonies per plate*							
		Unheated serum				Serum heated 56°C, 30 min.			
		Before incub.		After 6-hrs.		Before incub.		After 6-hrs.	
Dilution		0	1-10	0	1-10	0	1-10	0	1-10
Date	Dog No.								
9-27-62	140F	inn+	24	1	0	inn	19	300+	8
		inn	18	2	0	inn	28	300+	11
9-27-62	140G	inn	33	17	0	inn	30	inn	98
		inn	38	12	0	inn	36	inn	100
9-27-62	141I	inn	28	30	6	inn	34	inn	78
		inn	30	32	2	inn	48	inn	90
10-4-62	137D	inn	80	70	2	inn	85	inn	90
		inn	60	80	4	inn	70	inn	98
10-4-62	141G	inn	50	88	13	inn	58	inn	78
		inn	62	90	6	inn	66	inn	80
10-4-62	143C	inn	52	17	1	inn	75	inn	66
		inn	37	43	0	inn	70	inn	72
10-11-62	136D	inn	96	150	10	inn	119	inn	77
		inn	90	180	14	inn	129	inn	96
10-11-62	137E	inn	140	110	12	inn	130	inn	116
		inn	145	115	6	inn	122	inn	120
10-11-62	141H	inn	110	125	3	inn	96	inn	140
		inn	140	115	13	inn	100	inn	135
10-17-62	135E	inn	44	60	7	inn	66	inn	96
		inn	41	55	4	inn	58	inn	90
10-17-62	137F	inn	73	75	14	inn	46	inn	66
		inn	54	80	6	inn	50	inn	78
10-17-62	137B	inn	38	58	10	inn	60	inn	99
		inn	44	50	3	inn	56	inn	84

Experimental conditions: 0.5 ml serum, 0.16 ml bacterial suspension diluted 10^{-2}, 0.32 ml Kolmer's saline.

*Plate inoculated with 0.1 ml.

+inn = innumerable.

sera were inactivated by heating to 56°C. The addition of 0.16 ml of a 1-10 dilution of commercial guinea pig complement did not restore activity. This amount of complement hemolyzed sheep cells in the presence of hemolysin. It is still possible, however, that the activity might be in the complement supplied by the dog serum, since Dingle et al.[10] reported that guinea pig complement would not reactivate heated anti influenzae horse sera, but human complement would.

Dog serum does have a factor with the properties of complement found in other species, that is: the ability to hemolyze sheep cells in the presence of hemolysin and lability to heat[3]. This hemolytic activity was also determined in our laboratory with the serum obtained from the beagles used in the preceding experiments.

3. Comparison to Other Antibacterial Factors

Antibacterial factors for Gram negative organisms in human serum which were heat labile and which were not reactivated by the addition of guinea pig complement have been reported by Pillemer et al.[11], Pindak[6], and Waisbren and Brown[8]. Pillemer's group called the factor "properdin" and studied it in other species, but did not include the dog. Pindak presented evidence that the activity he studied was different from properdin. The anti-E. coli factor found in beagle's serum is similar to these previously reported bactericidins from human serum in these two properties. Further studies to characterize it as a component of complement, lysozyme, a "normal" antibody or properdin as attempted by others[12] were not done. We were interested only in finding a test for the bactericidal activity of the serum of the beagle which could be used, along with other tests, to evaluate the physiological effects of exposure to radiation.

TABLE 3

EFFECT OF COMPLEMENT ON THE BACTERICIDAL ACTIVITY OF SERUM FROM BEAGLES TOWARD *E. COLI*

	Number of colonies per plate*			
	Inactivated complement		Active complement	
	E. coli #4157			
Incubation period	0 hour	6 hour	0 hour	6 hour
Dog number				
Pool A**	inn	2	inn	2
Pool B	200+	3	200+	0
4	88	40	60	30
B4	125	2	150	20
15	100	67	80	38

Experimental conditions: 0.25 ml serum, 0.16 ml complement$_{-1}$ (commercial guinea pig complement diluted 10_{-3}), 0.08 ml bacterial suspension diluted 10^{-3}.

*Plates inoculated with 0.1 ml.

**Two dogs in each pool.

SUMMARY

The bactericidal activity of fresh serum from healthy beagles was determined. Serum was incubated with measured numbers of four different species of bacteria for six hours, after which the number of bacteria was again determined. The bacteria were counted by means of spread plates. No activity was found for B. subtilis, Pr. mirabilis, or Ps. aeruginosa. Serum from 12 dogs was found to be bactericidal to E. coli.

The activity was destroyed by heating to 56 °C for 30 minutes. It was not restored by adding guinea pig serum (complement), nor did the addition of complement to the fresh dog serum enhance its activity.

Since the bactericidal activity of the serum of the beagle is part of its immune mechanism and may be destroyed or enhanced by radiation, it could be a factor contributing to the physiological state of the animal after inhalation of radioactive fission products.

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